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Partial Deoxygenation of Semen Extender Minimizes Post-thaw Damages and Improves Freezability of Crossbred Bull Spermatozoa

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ABSTRACT

Background: Oxidative stress occurs when oxygen or oxygen derived oxidants exceed antioxidants and become responsible for poor post-thaw semen quality during the cryopreservation process. Therefore, the present study was designed to investigate the effects of dissolved oxygen (DO) levels (4, 6 and 8 ppm) in semen extender on freezability of crossbred bull spermatozoa. As the gap between 4 and 8 ppm existed in earlier studies, DO of 6 ppm in semen extender was further standardized and the effects were compared with other respective groups to contemplate any improvement in post-thaw semen quality.

Methods: For the experiment, Tris-egg Yolk-Glycerol (TYG) extender was partially deoxygenated by nitrogen gassing @ 2-3 bubbles/ sec at 34°C for 0, 16, 12 and 9 minutes to obtain DO levels of 11.7 ppm (Group-I/control), 4 ppm (Group-II), 6 ppm (Group-III) and 8 ppm (Group IV), respectively and collected semen samples were diluted with these extender groups to have 80×10^6 spermatozoa/ ml of the extender. Semen samples were evaluated for individual progressive motility (IPM), lipid peroxidation (LPO), total antioxidant capacity (TAC), plasma membrane integrity, acrosomal integrity and apoptotic changes at different stages of cryopreservation.

Result: At the post-thaw stage, progressive motility was greater ($p<0.05$) in Group II compared to Group I and the least reduction from the post-dilution to the post-thaw stage was observed in Group II. In comparison to Group I, Groups II, III and IV showed lesser ($p<0.05$) MDA production with Group II having greater ($p<0.05$) TAC concentration than other groups at the post-thaw stage. A declining trend was observed in membrane integrity as DO levels increased from 4 ppm to 11.7 ppm. Acrosomal integrity did not differ among treatment groups, but, found to be higher ($p<0.05$) than the control group. Per cent viable spermatozoa was greater ($p<0.05$) in Group II than Group I and vice versa for necrotic spermatozoa as assessed by Annexin V^{FITC}/PI staining. In conclusion, reducing the DO level to 4 ppm before cryopreservation improved the freezability by reducing oxidative stress and apoptotic changes while, above 4 ppm tended to lower it. An appreciable improvement in freezability can be seen at 6 ppm of DO, but, not up to that extent as observed at 4 ppm.

Key words: Annexin V^{FITC}/PI assay, CFDA/PI, Cryopreservation, Dissolved oxygen, Giemsa, Oxidative stress, Spermatozoa.

INTRODUCTION

Semen dilution, cooling, freezing and thawing exposes the spermatozoa to a number of stressful events. The first change that the spermatozoa have to experience is cold shock, which causes phase transition of the lipids in the membrane system (Woelders, 1997; Watson, 2000; Medeiros *et al*., 2002). A second change takes place when water is converted to ice at the time of freezing *i.e*. below 0°C in the form of ice crystals. Depending upon the freezing rate, either mechanical stress or osmotic stress causes structural and functional damages to spermatozoa. Thirdly, during the process of thawing, decrystallization of ice and reverse osmotic effects lead to the swelling and lysis of the spermatozoa (Kumar *et al.,* 2019b). Even though the above deleterious events of freezing have been controlled to a great extent by the addition of egg yolk and glycerol, there is still a poor recovery rate due to poor freezability of crossbred bull spermatozoa (Ghosh *et al.,* 2007; Panmei *et al.,* 2016).

The role of reactive oxygen species (ROS) and consequent oxidative stress in lowering the cryo-survivability of spermatozoa has been mentioned elsewhere (Pande *et al.,* 2019; Kumar *et al.,* 2019b). Bull spermatozoa are exposed to aerobic condition during processing before freezing and are highly susceptible to the effects of ROS due to the lack of sufficient endogenous antioxidants (Foote *et al.,* 2002). Excessive ROS production during the freezing-thawing

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process causes fatty acid peroxidation of membrane phospholipids and, thus, affects spermatozoal function (De Lamirande and Gagnon, 1995). It also leads to premature capacitation, tyrosine phosphorylation and apoptosis like changes which compromises fertilizing ability of spermatozoa *in-vivo* (Anzar *et al*., 2002; Martin *et al*., 2004; Kadirvel *et al*., 2012). Various enzymatic, non-enzymatic and plant based antioxidants have been used to tackle the problem of oxidative stress during the cryopreservation process as reviewed earlier (Bansal and Bilaspuri, 2011; Amidi *et al.,* 2016; Kumar *et al.,* 2019b). Another method for minimizing the oxidative stress was lowering the oxygen tension *i.e.* partial deoxygenation of the semen extender. Three different approaches have been used *i.e.* flash frozen-thaw process using liquid nitrogen, nitrogen gas bubbling/gassing process (2-3 bubbles per second) using gaseous nitrogen and applying negative pressure to the flask containing diluent with the help of modified vacuum pump (Pande *et al.,* 2015; Mustapha *et al.,* 2017; Amin *et al.,* 2018; Balamurugan *et al*., 2018; Kumar *et al.,* 2018). Further refinement of DO level was indicated in nitrogen gassing method as a gap between the 4 ppm and 8 ppm existed. Therefore, to precisely determining the DO concentration for optimizing the post-thaw semen quality, 6 ppm Group was taken and compared with fellow DO level groups in the present study.

MATERIALS AND METHODS

The proposed study was conducted at the Germ-Plasm Centre, Division of Animal Reproduction, Indian Veterinary Research Institute, Izatnagar, Bareilly, India. The institute is located at an altitude of 564 feet above the mean sea level, at latitude of 28°North and longitude of 79°East. The place has a subtropical climate and experiences both the extremes of hot and cold weather conditions with the relative humidity ranging between 15 to 85% in different months of the year. All the experimental procedures and protocols were duly approved by the Institute's Animal Ethics Committee.

Experimental animals

Three healthy crossbred bulls (4 to 6 years of age) maintained under same managemental conditions at Germ-Plasm Centre were utilized during one year of research. These bulls are used in routine frozen semen production at the centre.

Deoxygenation of semen extender

The Tris-egg Yolk-Glycerol (TYG) extender (Tris 3.028 g, citric acid 1.675 g, fructose 1.25 g, egg yolk 20% v/v, glycerol 7% v/v, benzyl penicillin 1000 IU/ml and streptomycin sulphate 1000 µg/ml, double distilled water 73 ml to make final volume of 100 ml) was used in the present study. Following the preparation of extender, it was kept at a constant temperature (34°C) in a water bath. Then, the extender was divided into 4 test tubes of 50 ml capacity and two of these tubes were deoxygenated using nitrogen gas at the rate of 2-3 bubbles per second at a constant temperature (34°C) for 9 min and 16 min to obtain 8 ppm (Group IV) and 4 ppm (Group II) dissolved oxygen levels, respectively as standardized by Mustapha *et al.* (2017). The protocol was standardized for the new treatment group (Group-III; 6 ppm), using the same trial and error method. Precaution was taken to avoid entry of environmental oxygen by covering the tubes with Para film®. In control (Group I), extender without nitrogen gassing was having DO level of ≈11.7 ppm at 34°C (Fig 1). The DO meter (Century, CD 501, India) was used to measure the DO levels of respective groups.

Semen processing and preservation

Semen collection, dilution, equilibration, freezing and thawing were done as per method described by Kumar *et al.* (2019a), (Fig 1). Individual progressive motility was assessed at the post-dilution, pre-freeze and post-thaw stages. Oxidative stress parameters (TAC and MDA) were assessed at the pre-freeze and post-thaw stages. Acrosome integrity, membrane integrity and apoptosis status of spermatozoa were evaluated at the post-thaw stage only (Fig 1).

Semen evaluation

Individual progressive motility

The individual progressive motility of the diluted semen was assessed by placing a small (10 µl) drop of diluted semen on a pre-warmed glass slide (37°C) and allowing it to spread uniformly under the cover slip (18×18 mm). About ten widely-spaced fields were examined to provide an estimate of the percentage of progressive motile spermatozoa at 200X using light microscope.

Plasma membrane integrity

The CFDA-PI (Carboxy fluorescein diacetate- Propidium iodide) was used to evaluate the plasma membrane integrity of spermatozoa as described by Singh *et al*. (2016). One hundred ìl of thawed semen sample was washed twice using PBS having temperature 37°C by centrifugation at 170 g for 10 min. After removal of supernatant, the final volume was made up to 200 µl with PBS having temperature 37°C, then 5 µl of CFDA (0.5 mg/ml) kept at 37°C was added into sperm suspension and incubated at 37°C for 15 min in the dark. Then, 2.0 µl of PI (0.3 mg/ml) was added and further incubated for 2 min. After that, 10 µl of this suspension was used to prepare a smear on a grease-free slide. A cover slip (22×50 mm) was placed after putting a drop of anti-fade solution DABCO 33-LV on the stained smears, lightly pressed and edges were sealed with colorless nail varnish. Slides were examined under fluorescent microscope (MT6300, Meji Techno., Japan) at 400X magnification. A total of 200 spermatozoa were counted and percentages of spermatozoa with intact plasma membrane were determined.

Acrosomal integrity

Acrosome integrity was determined by using Giemsa stain

as per the method described by Watson (1975). A smear from a drop of frozen-thawed semen was prepared on a clean grease free glass slide and air dried. The smear was fixed in Hancock's fixative for 15 min. Then, fixed smear was washed in slow running water for 15 min and air dried. After drying, the smear was stained with Giemsa working solution for 180 min. Slides were then rinsed quickly in distilled water and air dried. After that, smear was examined at 1000X under oil immersion objective of the microscope and at least 200 spermatozoa were counted each slide.

Oxidative stress status

Estimation of lipid peroxidation (LPO)

Lipid peroxidation level of sperm pellet was measured by determining the malondialdehyde (MDA) production as per the method described by Buege and Aust (1978) and modified by Suleiman *et al.* (1996). After centrifugation, the spermatozoal pellet was resuspended in PBS (pH 7.2) to a concentration of 20×10^6 spermatozoa/ml. Then, 2 ml of thiobarbituric acid-trichloroacetic acid (TBA-TCA) reagent (15% w/v TCA, 0.375% w/v TBA and 0.25 N HCL) was added into this suspension. The mixture was treated in a boiling water bath for 1 hour. After cooling, the suspension was centrifuged at 3000 rpm for 10 min. The supernatant was then separated and absorbance was measured at 535 nm. The MDA concentration was determined by the specific absorbance coefficient (1.56×10^5 µmol/cm^3).

Estimation of total antioxidant capacity (TAC)

Total antioxidant capacity in seminal plasma was measured using ferric reducing/antioxidant power (FRAP) assay described by Benzie and Strain (1996). Briefly, 100 µl of extended seminal plasma was mixed with 3 ml of working FRAP reagent and absorbance (593 nm) was measured at 0 min after vortexing. Thereafter, samples were placed at 37°C in a water bath and absorption was again measured after 4 min. The working FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2, 4, 6-tripyridyl-*s*-triazine) in 40 mM HCL and 20 mM $FeCl_3.6H_2O$ (Ferric chloride hexahydrate) in a 10:1:1 ratio. Ascorbic acid

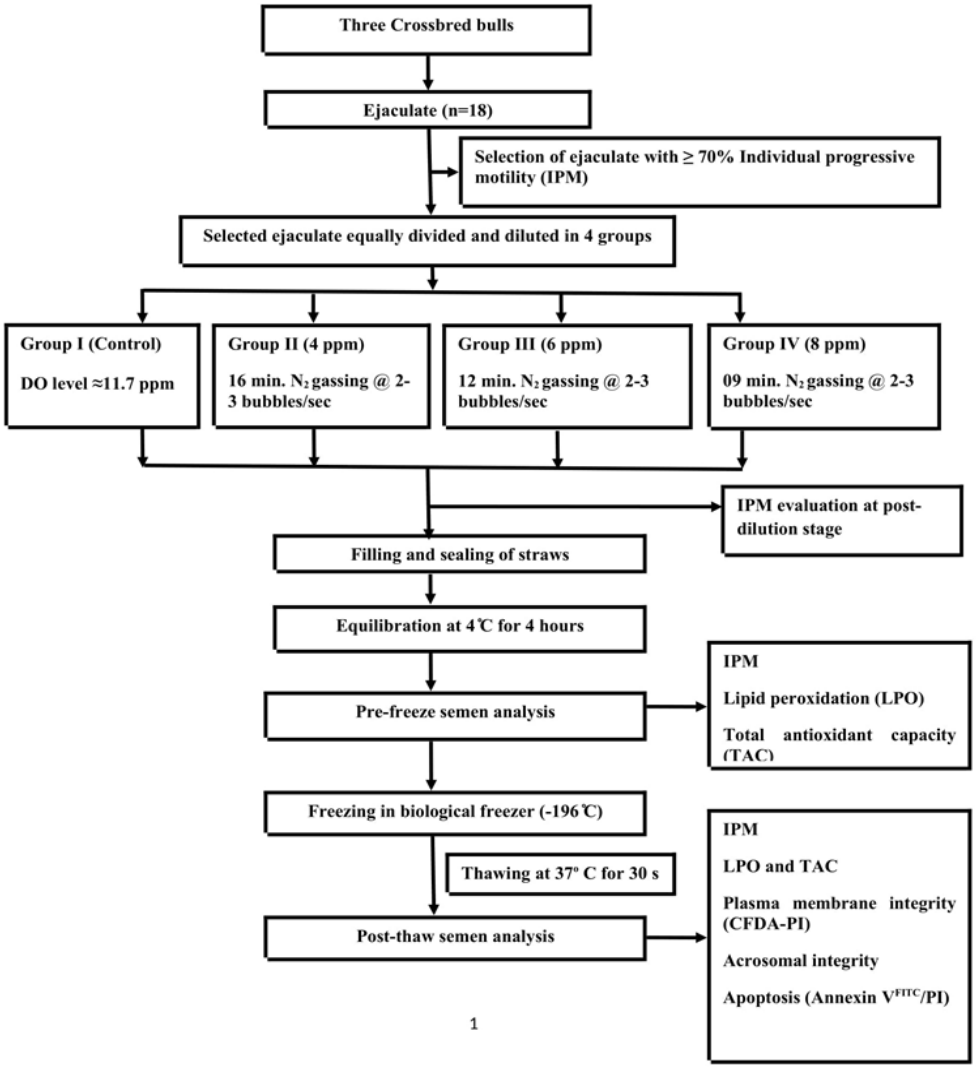

Fig 1: Schematic representation depicting brief outline of materials and methods used in research work.

standards were processed in the same way as the seminal plasma sample. Three ml of working FRAP solution was used as blank.

Apoptosis assessment

It was performed using Annexin V^{FITC}/PI stain as described by Anzar *et al.* (2002) with slight modifications. Washing of the semen was done by adding HEPES buffer (Sigma-Aldrich, USA) and centrifugation at 800 rpm for 5 min. After discarding the supernatant, the pellet was resuspended in 500 µl HEPES buffer. 10 µl Annexin V (50 µg/ml) was added in 100 µl of spermatozoal suspension and incubated for 10 minutes in the dark at 37°C. Then, 10 µl PI (100 µg/ml) was added in it and centrifuged at 800 rpm for 3 min. The supernatant was discarded and a thin smear was made from the pellet on a clean grease free slide. An anti-fading agent DABCO 33- LV was added to the smear before applying cover slip (22×50 mm) on it. Spermatozoa were observed under fluorescent microscope at 1000X and at least 200 spermatozoa were counted. Four populations of spermatozoa were identified *i.e.* early Apoptotic (Annexin V^{FITC+}/PI^{-}), early necrotic/late apoptotic (Annexin V^{FITC+}/PI^{+}), necrotic (Annexin V^{FITC-}/PI^{+}) and fully viable (Annexin V^{FITC-}/PI^{-}).

Statistical analysis

The generated data from the experiment was analysed using ANOVA. Mean values among control and treatment groups and at different stages were compared by Tukey's multiple comparison test.

RESULTS AND DISCUSSION

Oxidative stress is one of the important factors responsible for poor post-thaw semen quality which is experienced by spermatozoa during the freezing-thawing process (Kumar *et al.,* 2019b). It is mainly inflicted by oxygen or oxygen derived oxidants *i.e.* ROS. As oxygen was directly or indirectly involved in execution of this condition, one strategy was to minimize the dissolved oxygen in sperm microenvironment *i.e.* semen extender in order to reduce the oxidative stress and to improve the post-thaw semen quality (Fig 2). Therefore, nitrogen gassing method was developed and after nitrogen gassing at 2-3 bubbles/sec for 40, 16 and 9 minutes in semen extender, the DO levels were approximately 2 ppm, 4 ppm and 8 ppm, respectively (Mustapha *et al.,* 2017). The present study was based upon the certainty that a gap between 4 ppm and 8 ppm existed, therefore, further refinement of DO concentration was required to optimize the level of dissolved oxygen in semen extender and to compare the improvement in freezability of spermatozoa with other DO levels. Using the trial and error method, the time required to achieve approximately 6 ppm of dissolved oxygen in the extender by nitrogen gassing was determined to be 12 minutes. In control group without N_2 gassing, the DO level was approximately 11.7 ppm as earlier reported (Mustapha *et al.,* 2017).

The per cent progressive motile spermatozoa was greater ($p<0.05$) in Group II compared to Group I and no significant difference was observed among the Groups II, III and IV as well as among the Groups I, III and IV at the pre-freeze and post-thaw stages (Table 1). There was significant ($p<0.05$) reduction in the per cent progressive motile spermatozoa from the post-dilution to the post-thaw stage (Pathak *et al.,* 2020), in which the least reduction in motility was observed in Group II (≈40.07%) and the highest reduction was observed in Group I (≈50.18%, Table 1). The lower motility in the control group compared to the treatment groups indicated that the higher DO concentration in the control group was negatively correlated with the progressive motility suggesting higher oxidative metabolism, higher concentrations of ROS generation and consequent damage to the motility apparatus of the spermatozoa (Kumar *et al.,* 2019a; Katiyar *et al.,* 2020). In treatment groups, DO levels higher than 4 ppm tended to have lesser percentage of progressive motile spermatozoa which might be due to increased oxidative metabolism as mentioned. Therefore, the findings indicated that at 4 ppm of DO, there was an

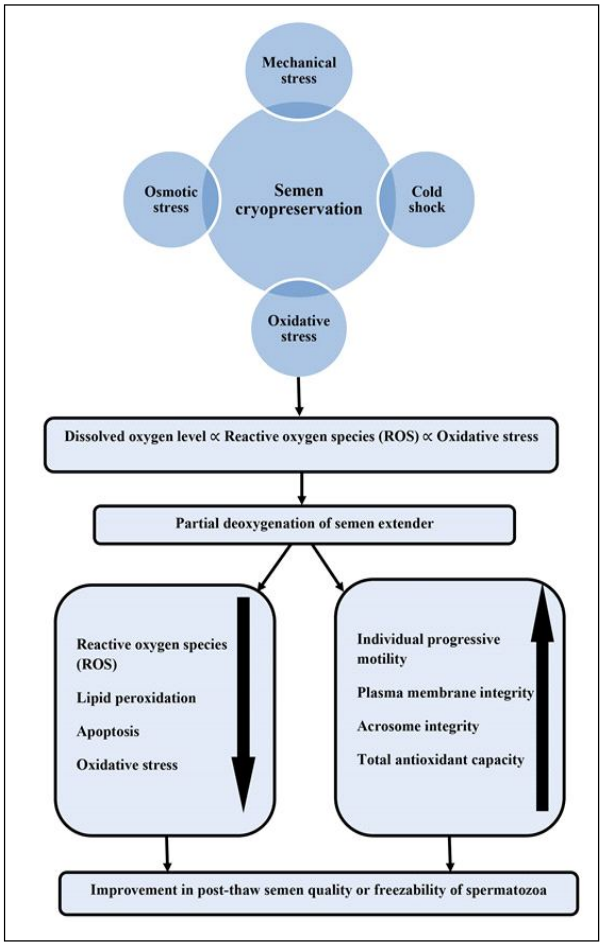

Fig 2: Schematic diagram showing various stresses related to semen cryopreservation process and partial deoxygenation of semen extender for improvement in sperm freezability.

optimum balance between ROS generation and metabolic activity of spermatozoa.

The MDA production was lesser ($p<0.05$) in Group II as compared to Group I at the pre-freeze stage, but, no significant difference was recorded among the Groups II, III and IV as well as among the Groups I, III and IV (Table 1). At the post-thaw stage, MDA production was lowest ($p<0.05$) in Group II and highest ($p<0.05$) in Group I (Table 1). However, no significant difference was recorded between the Groups III and IV, but, these values were greater ($p<0.05$) than Group II and lesser ($p<0.05$) than Group I. Group II had highest ($p>0.05$) mean TAC value among all the groups at the pre-freeze stage (Table 1). The TAC values were greater ($p<0.05$) in treatment groups as compared to control group, but, no significant difference was observed among the treatment groups at the post-thaw stage (Table 1). Lipid peroxidation of plasma membrane increases after freezing and thawing, which results in metabolic changes as well as loss of motility, membrane integrity and fertilizing capability of spermatozoa (Agarwal and Prabakaran, 2005). As DO was found to be responsible for ROS production and subsequently lipid peroxidation (Kumar *et al.,* 2018), partial deoxygenation of semen extender reduced lipid peroxidation during semen cryopreservation which was indicated by lesser MDA production at the post-thaw stage in the present study (Fig 2). It was more pronounced in 4 ppm group compared to other treatment groups at this stage. The determination of total antioxidants in seminal plasma of bull is predictive tool for the evaluation of oxidative stress in frozen thawed sperm (Gürler *et al*., 2015). The lowest TAC value observed in the control group at the post-thaw stage might be due to greater utilisation of available antioxidants to scavenge the free radicals generated. Higher TAC concentrations in treatment groups possibly indicated lesser free-radical formation due to suppressed oxidative metabolism at lower DO concentrations (Kumar *et al.,* 2018).

At the post-thaw stage, per cent membrane-intact spermatozoa was greater ($p<0.05$) in Group II as compared to Groups I and IV, but, no significant difference was observed between Groups II and III; III and IV and I and IV (Table 1). A declining trend was observed in the per cent membrane intact spermatozoa as DO concentrations increased from 4 ppm to 11.7 ppm (Control) and it indicated a possible loss of membrane integrity of spermatozoa as the concentration of DO increased in the semen extender. It might be due to increased ROS mediated freeze-thaw damages at higher DO concentrations, which was reflected in terms of lower membrane intactness at the post-thaw stage (Fig 2).

The mean per cent of spermatozoa with intact acrosome was greater ($p<0.05$) in Groups II, III and IV as compared to Group I and no significant difference was observed among the treatment groups at the post-thaw stage (Table 1). Numerically, the highest per cent of spermatozoa with intact acrosome was observed in Group II and the lowest in Group I (Table 1). Two of the most important physiological roles of

Table 1: Effect of partial deoxygenation of semen extender on semen quality parameters of crossbred bull at different cryopreservation stages (Mean±S.E., n=18).

Parameters	Stage	Group I (Control)	Group II (4 ppm)	Group III (6 ppm)	Group IV (8 ppm)
Progressive motile spermatozoa (%)	Post-dilution	79.72 ± 0.75^{Aa}	79.72 ± 0.75^{Aa}	79.72 ± 0.75^{Aa}	79.72 ± 0.75^{Aa}
	Pre-freeze	65.56 ± 1.27^{Bb}	72.22 ± 0.92^{Ba}	69.44 ± 1.33^{Bab}	68.89 ± 1.25^{Bab}
	Post-thaw	39.72 ± 2.0^{Cb}	47.78 ± 1.52^{Ca}	43.06 ± 2.14^{Cab}	43.61 ± 1.93^{Cab}
MDA (µmol/ml)	Pre-freeze	0.470 ± 0.015^{Aa}	0.393 ± 0.012^{Ab}	0.421 ± 0.016^{Aab}	0.425 ± 0.014^{Aab}
	Post-thaw	0.898 ± 0.013^{Ba}	0.674 ± 0.012^{Bc}	0.745 ± 0.009^{Bb}	0.781 ± 0.016^{Bb}
TAC (nmol/ml)	Pre-freeze	651.32 ± 19.99^{Aa}	711.30 ± 21.34^{Aa}	688.98 ± 11.24^{Aa}	682.47 ± 10.24^{Aa}
	Post-thaw	386.80 ± 17.80^{Bb}	462.58 ± 18.24^{Ba}	413.76 ± 19.92^{Bab}	417.48 ± 18.28^{Bab}
Plasma membrane integrity (%)	Post-thaw	42.16 ± 1.33^{c}	51.95 ± 1.10^{a}	47.40 ± 1.20^{ab}	46.01 ± 1.39^{bc}
Acrosomal integrity (%)	Post-thaw	54.84 ± 1.06^{b}	67.15 ± 1.55^{a}	62.37 ± 1.64^{a}	61.74 ± 1.38^{a}
Apoptosis (Annexin V^{FITC}/PI) (Post-thaw stage)	Viable	33.78 ± 1.13^{b}	41.55 ± 1.89^{a}	36.14 ± 1.31^{ab}	36.26 ± 1.51^{ab}
	Early apoptotic	6.96 ± 0.48	11.06 ± 0.28	10.69 ± 0.39	11.44 ± 0.31
	Early necrotic/late apoptotic	40.45 ± 1.12	37.03 ± 1.56	38.98 ± 1.06	40.63 ± 0.99
	Necrotic	18.81 ± 2.18^{a}	10.36 ± 0.75^{b}	14.19 ± 0.69^{b}	11.67 ± 0.81^{b}

Means bearing different superscripts in small letters (a, b, c) in a row differ significantly ($p<0.05$).
Means bearing different superscripts in capital letters (A, B, C) in a column differ significantly ($p<0.05$); n= Number of ejaculates.

ROS are the induction of capacitation and the acrosomal reaction (Satorre *et al*., 2007; Aitken, 2017). These are vital for fertilization, their early induction during the freezing and thawing process is unfavourable to the longevity of spermatozoa in the female reproductive tract (Medeiros *et al.,* 2002). In the present study, treatment groups showed better acrosomal integrity and this finding suggests that there was an obvious negative effect of higher DO levels in the extender on the acrosomal integrity of the spermatozoa (Fig 2). It seemed to be a balance between the physiological and pathological effects of ROS reflected by greater percentage of acrosome intactness in 4 ppm group followed by 6 and 8 ppm groups' than the control group.

Bull fertility is highly correlated to the percentage of viable spermatozoa in semen as detected by the Annexin V^{FITC}/PI assay (Anzar *et al*., 2002), because it also detects early apoptotic spermatozoa which are motile and possess intact membrane but are in a state of early apoptotic changes characterised by phosphatidylserine (PS) externalisation and low mitochondrial potential (Miki *et al*., 2004; Martin *et al*., 2007). Cryopreservation acts as an apoptotic mechanism inducer in bovine spermatozoa, where the earliest (PS translocation) but also the late features (DNA fragmentation) of cells undergoing apoptosis occur (Martin *et al*., 2004). Increase in the apoptotic and necrotic spermatozoa and decrease in the viable spermatozoa population during the freezing-thawing process has been observed in cattle (Anzar *et al*., 2002; Martin *et al*., 2004) and buffalo (Kadirvel *et al*., 2012). In addition, oxidative stress is one of the major factors responsible for apoptotic changes in frozen-thawed semen (Said *et al*., 2010). In the present study, per cent viable spermatozoa was significantly ($p<0.05$) greater in Group II compared to Group I at the post-thaw stage (Table 1). Early apoptotic spermatozoa population didn't differ significantly among all the four groups. However, numerically lowest percentage of early apoptotic spermatozoa was observed in the Group I, while in the treatment groups, close values were observed which were greater than Group I (Table 1). The per cent early necrotic/late apoptotic spermatozoa were least in Group II and no significant difference was observed among all the groups. The per cent necrotic spermatozoa was greater ($p<0.05$) in the Group I as compared to Groups II, III and IV, but, no significant difference was observed among the treatment groups at this stage (Table 1). The 4 ppm group had greater viable spermatozoa and lesser necrotic spermatozoa compared to the control group which could be due to the beneficial effect of reduced DO concentration before cryopreservation. It might be possible that the spermatozoa in the 4 ppm DO group experienced lower oxidative stress during the freezing-thawing process resulting in better membrane integrity (Fig 2).

In this study, the per cent viable sperm detected by AnnexinV^{FITC}/PI was lower than those detected by CFDA/PI because CFDA/PI cannot differentiate between early apoptotic and non-apoptotic viable spermatozoa as early apoptotic ones also possess an intact membrane (Anzar *et al*., 2002). Eosin-negrosin staining tends to overestimate viability in comparison to fluorescent based stains as earlier reported (Brito *et al.,* 2003).

CONCLUSION

Nitrogen gassing at 2-3 bubbles/sec was done to bring the DO level from ≈11.7 ppm to ≈6 ppm in about 12 minutes at 34°C. Reducing the DO level of extender to 4 ppm improved the freezability by reducing oxidative stress and apoptotic changes in bull spermatozoa while increasing the DO concentrations above 4 ppm tended to lower it. An appreciable improvement in freezability was observed when DO level of extender was brought to 6 ppm, but, not up to that extent when it was brought to 4 ppm before cryopreservation.

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Conflict of interest

The authors declare no conflict of interest.

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